AWARD NUMBER: W81XWH-15-1-0382

TITLE: Selective Activation of a Perforin-Granzyme B Fusion Protein Toxin by PSA as Therapy for Metastatic Prostate Cancer

PRINCIPAL INVESTIGATOR: Samuel Denmeade

RECIPIENT: Johns Hopkins University
Baltimore, MD 21218

REPORT DATE: October 2016

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT:

Approved for public release; Distribution unlimited

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	**2. REPORT TYPE**	**3. DATES COVERED** *(From - To)*
October 2016	Annual	30 Sep 2015 - 29 Sep 2016

4. TITLE AND SUBTITLE

Selective Activation of a Perforin-Granzyme B Fusion Protein Toxin by PSA as Therapy for Metastatic Prostate Cancer

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-15-1-0382

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Samuel R Denmeade; email-denmesa@jhmi.edu

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Department of Pharmacology and Molecular Sciences
The Johns Hopkins University
600 North Wolfe Street
Baltimore, MD 21287

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S Army Medical Research And Materiel Command
Fort Detrick MD 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for public release; Distribution unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT Protein toxins represent a class of agents that can kill cells in a proliferation independent manner. Many such proteins, derived primarily from bacterial sources, have been identified that are highly potent cytotoxins. While this approach has great potential, the major limitation is the fact that the protein toxin proves to be highly immunogenic and not amenable to repeated dosing to achieve maximal antitumor effect. This obstacle can be overcome through the use of human protein toxins. The goal of this proposal, therefore, is to develop a targeted cytotoxic agent that can selectively kill both proliferating and non-proliferating prostate cancer cells within a metastatic site without significant host toxicity. To achieve this goal, we propose to modify Granzyme B, the major cell-killing components present in cytotoxic T lymphocyte (CTL) granules to a form that is selectively targeted to prostate cancer cells. Granzyme B is a zymogen that must be proteolytically activated and then must penetrate cell membranes to proteolytically activate intracellular pro-apoptotic factors. We describe a targeting approach that recapitulates this dual activation but redirects it to prostate cancer cells. We will replace the native two amino acid propeptide of granzyme B with a peptide recognized as a substrate by PSA. To facilitate granzyme B internalization, we will couple a potent small molecule inhibitor of PSMA to the C-terminus of granzyme B. This granzyme B toxin will only be activated in the prostate cancer microenvironment while remaining inactive against normal tissues lacking both PSA and PSMA

15. SUBJECT TERMS
Granzyme B, PSA, PSMA, protoxin, protease, zymogen

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON**
					USAMRMC
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**			**19b. TELEPHONE NUMBER** *(include area code)*
U	U	U	UU	17	

Standard Form 298 (Rev. 8-98)
Prescribed by ANSI Std. Z39.18

TABLE OF CONTENTS

1. INTRODUCTION:

While prostate cancer can grow slowly, it is not curable with current therapies once it has metastasized outside of the prostate gland. Since prostate cancer cells have a remarkably low proliferation rate, novel therapies designed to selectively target and kill non-proliferating prostate cancer could be highly effective in this disease. Protein toxins represent a class of agents that can kill cells in a proliferation independent manner. Many such proteins, derived primarily from bacterial sources, have been identified that are highly potent cytotoxins. Attempts have been made to redirect the potent, but non-specific cytotoxicity of these toxins to produce tumor or tissue specific cell killing. This is typically accomplished through the creation of fusion proteins in which a cell-type specific ligand (e.g. cytokines) or antibody is fused to the toxic portion of the bacterial protein. While this approach has great potential, the major limitation of this approach is the fact that, in all cases described, the protein toxin proves to be highly immunogenic and not amenable to repeated dosing to achieve maximal antitumor effect. This obstacle can be overcome through the use of human protein toxins. The goal of this proposal, therefore, is to develop a targeted cytotoxic agent that can selectively kill both proliferating and non-proliferating prostate cancer cells within a metastatic site without significant host toxicity. To achieve this goal, we propose to modify Granzyme B, the major cell-killing components present in cytotoxic T lymphocyte (CTL) granules to a form that is selectively targeted to prostate cancer cells. Granzyme B is a zymogen that must be proteolytically activated and then must penetrate cell membranes to proteolytically activate intracellular pro-apoptotic factors. We describe a targeting approach that recapitulates this dual activation but redirects it to prostate cancer cells. We will replace the native two amino acid propeptide of granzyme B with a peptide recognized as a substrate by PSA. To facilitate granzyme B internalization, we will couple a potent small molecule inhibitor of PSMA to the C-terminus of granzyme B. This granzyme B toxin will only be activated in the prostate cancer microenvironment while remaining inactive against normal tissues lacking both PSA and PSMA.

2. KEYWORDS:

Granzyme B, PSA, PSMA, protoxin

3. ACCOMPLISHMENTS:

What were the major goals of the project?

The major goals of the project are:
Aim 1: Synthesize, purify, and characterize modified GZMB proteins and analyze for PSA activation and PSMA binding and stability in mouse and human plasma.
Aim 2: Demonstrate the selectivity of modified GZMB proteins against PSA/PSMA producing prostate cancer cells but not PSA/PSMA negative non-prostate cancer cells in vitro.
Aim 3: Evaluate toxicity and efficacy of the lead modified GZMB proteins in vivo against PSA-producing prostate cancer xenografts.

What was accomplished under these goals?

Major activities/specific objectives

Aim1 Activities

1) Generated multiple mutant forms of Granzyme B in which the native pro-domain has been replaced by pro-domains selectively recognized as a substrate for PSA
2) Produced additional multiple mutant forms of Trypsin as a positive control in which the native pro-domain has been replaced by pro-domains selectively recognized as a substrate for PSA
3) Demonstrated that PSA could hydrolyze PSA-Granzyme B to generate active Granzyme B protease
4) Demonstrated that PSA could hydrolyze PSA-trypsin to generate active trypsin protease
5) Performed Michaelis Menten enzyme kinetic studies to characterize PSA activation of these protoxins.
6) Generated a mutant form of Granzyme B containing a C-Terminal cysteine
7) Coupled a maleimide-containing PSMA inhibitor to the C-terminal cysteine of Granzyme B to generate a PSMA-targeted Granzyme B protein
8) Demonstrated PSMA-Granzyme B selectively binds to PSMA and becomes internalized into recycling endosome.

Aim 2 Activities

1) Evaluated toxicity of PSA-activated Granzyme B against PSA-producing (LNCaP, CWR22Rv1) and non-PSA producing (DU145) human prostate cancer cell lines in vitro.
2) Demonstrated that extracellular activation of Granzyme B can kill prostate cancer cells through effects on extracellular matrix.
3) Evaluated toxicity of PSA-activated Trypsin against PSA-producing (LNCaP, CWR22Rv1) and non-PSA producing (DU145) human prostate cancer cell lines in vitro.
4) Demonstrated selective uptake of PSMA-Granzyme B in PC-3 cells transfected to produce PSMA but no uptake in PC-3 transfected with vehicle control.
5) Generate fluorescent PSMA-Granzyme B to show specific uptake and concentration of protein in the recycling endosome.
6) Performed cytotoxicity assays with PSMA-Granzyme B against PSMA producing LNCaP and PSMA transfected PC3 vs. PSMA negative PC3 cells.

Aim 3 Activities

None

3) Significant Results

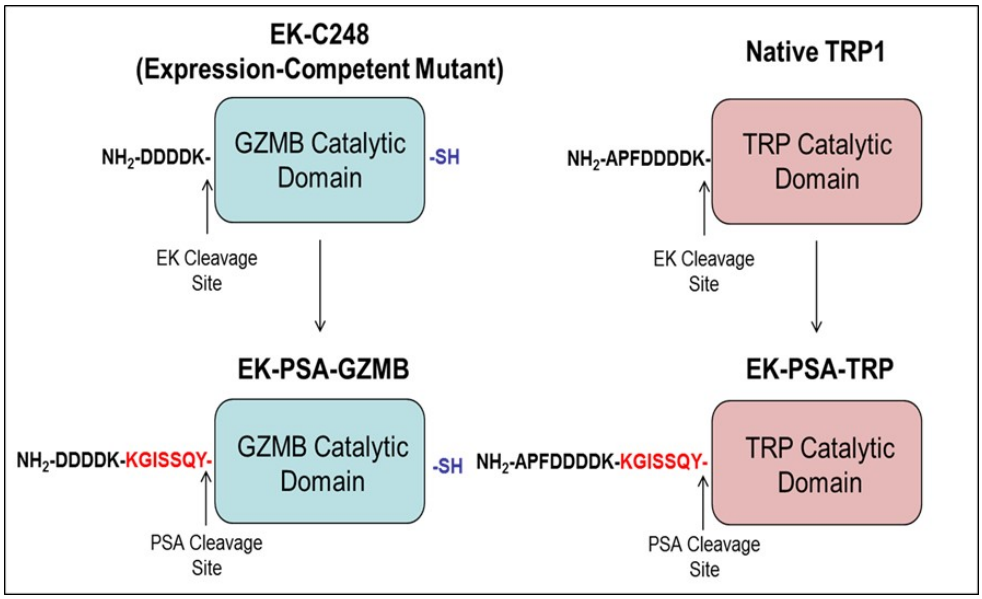
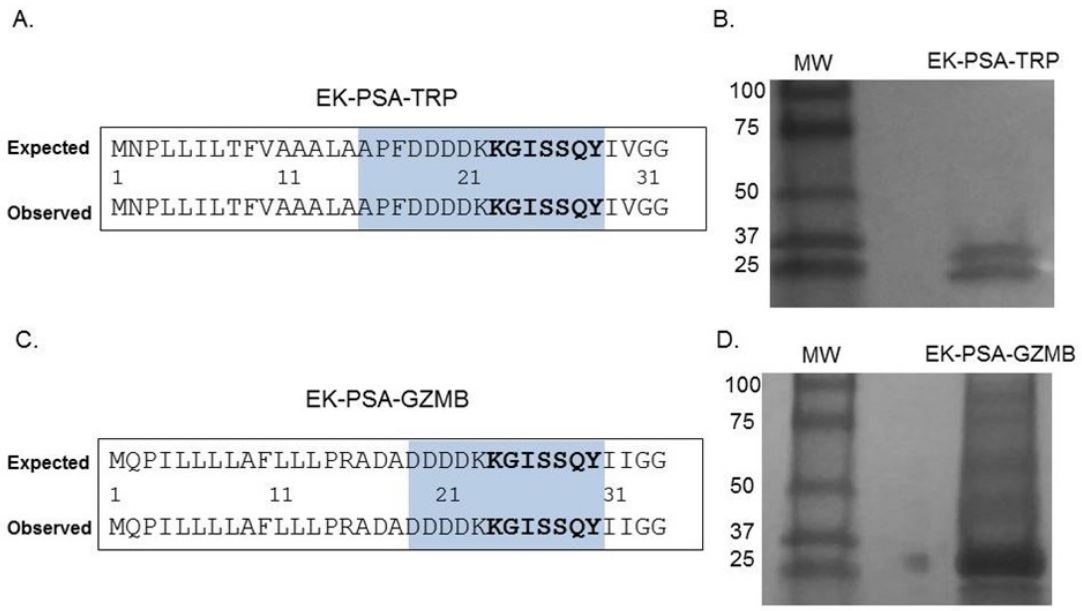
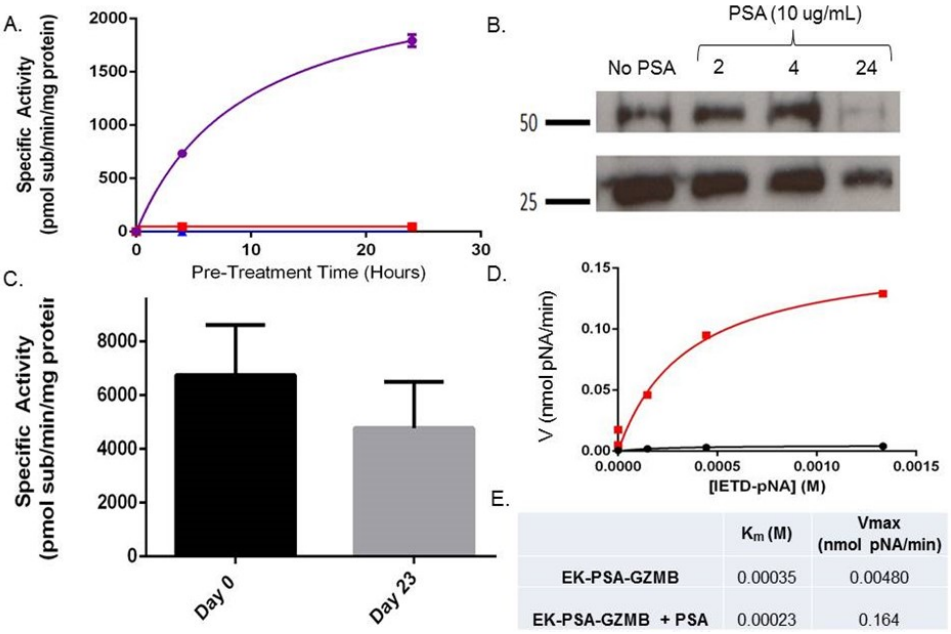


Figure 1: Schematic of EK-PSA-GZMB and EK-PSA-TRP pro-drugs. Mutations made to convert EK-activated GZMB into a PSA-activated zymogen (left) and WT Trypsin into a PSA-activated zymogen (right).

Figure 2. Cloning and Expression of PSA-Activated TRP and GZMB. Sanger DNA sequencing results of the mutated N-terminus of both EK-PSA-TRP (A) and EK-PSA-GZMB (C) genes. CBB-stained reducing SDS-PAGE gels with EK-PSA-TRP (B) and EK-PSA-GZMB (D). These results demonstrate that PSA-activated Trypsin and Granzyme B proteins have proper insert.





	K_m (M)	Vmax (nmol pNA/min)
EK-PSA-GZMB	0.00035	0.00480
EK-PSA-GZMB + PSA	0.00023	0.164

Figure 3: EK-GZMB-TRP is a PSA-activated Granzyme B mutant. Enzymatic hydrolysis of GPR-pNA of EK-PSA-GZMB (red), PSA (blue), and EK-PSA-TRP mixed with PSA (purple) pre-incubated for various times (A). Western blot analysis of EK-PSA-GZMB +/- PSA probed with an anti-DDDDK rabbit polyclonal antibody (B). Enzymatic activity of EK-PSA-GZMB incubated for various time points at 37 degrees after being pre-exposed to PSA (C). Michaelis-Menten enzyme kinetics of EK-PSA-GZMB (black) or EK-PSA-TRP with PSA (red) (D). Calculated enzymatic parameters of inactive and activated EK-PSA-GZMB.

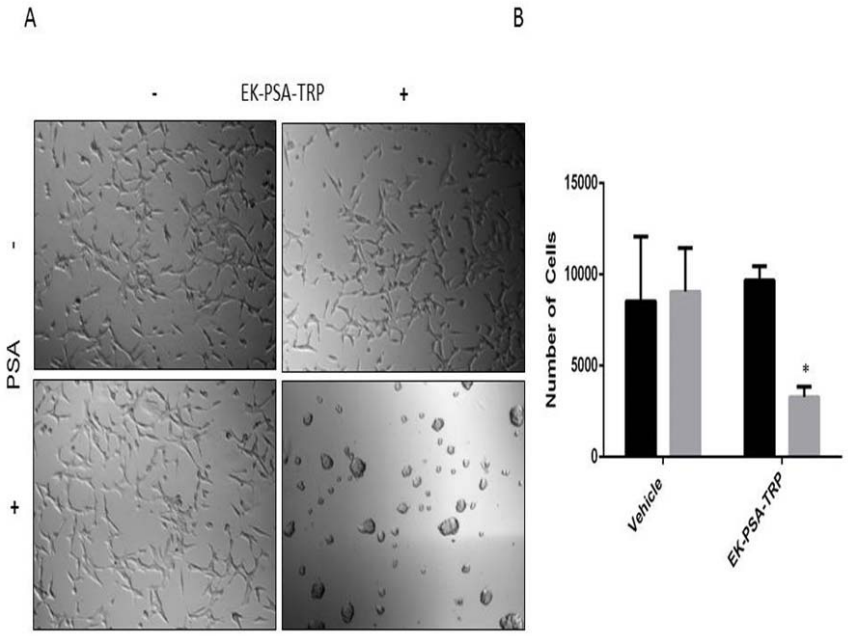


Figure 4: EK-PSA-TRP inhibits LNCaP cell growth selectively in the presence of PSA. Light microscopy images of LNCaP cells treated +/- 500 nM EK-PSA-TRP and +/- PSA at 10X magnification (A). MTT assay of LNCaP cells treated with either buffer or 500 nM EK-PSA-TRP and either control (black bars) or PSA-containing (grey bars) media after 5 days (B).

Figure 5: EK-PSA-TRP inhibits prostate cell growth selectively in the presence of PSA. Light microscopy images of LNCaP cells treated +/- 3 uM EK-PSA-GZMBand +/- PSA at 10X magnification (A). MTT assay of LNCaP (B), DU145 (C), CWR22 Rv1 (D), treated with either buffer or 3 uM EK-PSA-GZMB and either control (black bars) or PSA-containing (grey) media after 5 days.

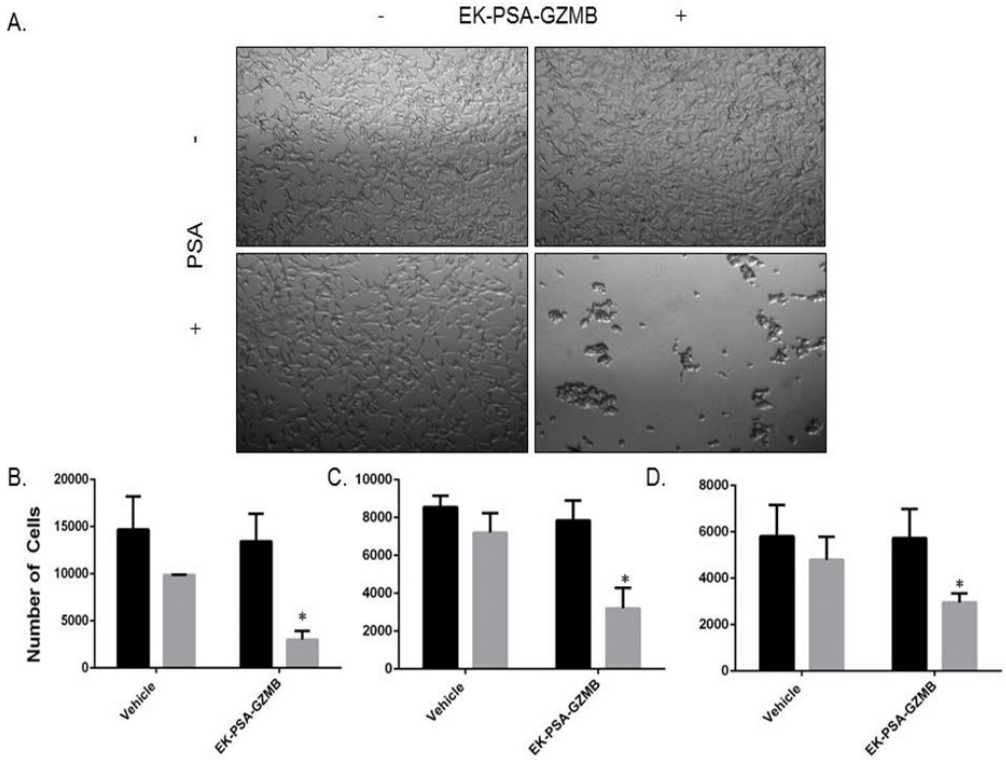


Figure 6: GZMB induces prostate cancer cell growth inhibition via damage to the ECM. Light microscopy images of LNCaP (top) and CWR22 Rv1 (bottom) were plated in serum free RPMI (B27 supplemented). in wells treated with buffer (left), media-containing serum (middle) or media with serum and 1 uM GZMB (right) and incubated for 48 hours at 37 degrees. Magnification is 10X (A). Quantitation of plated LNCaP (left) and CWR22 Rv1 (right) 48 hours after plating (B).

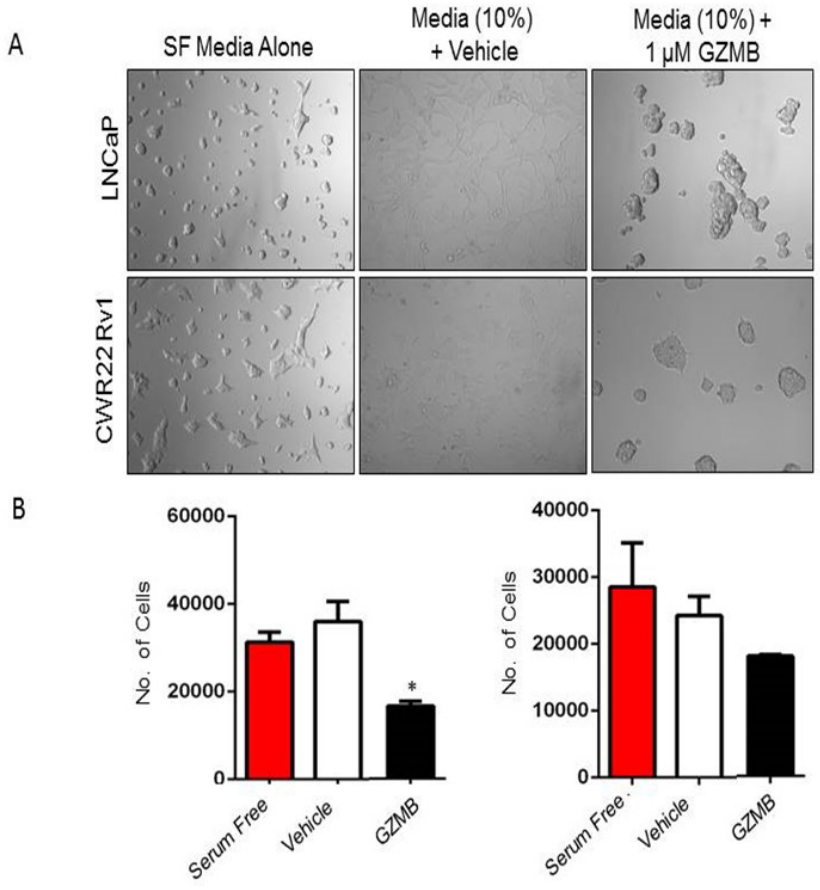
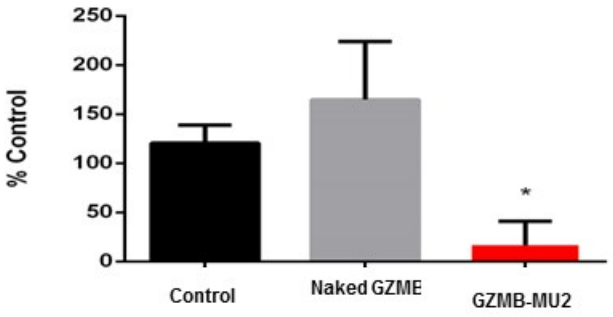
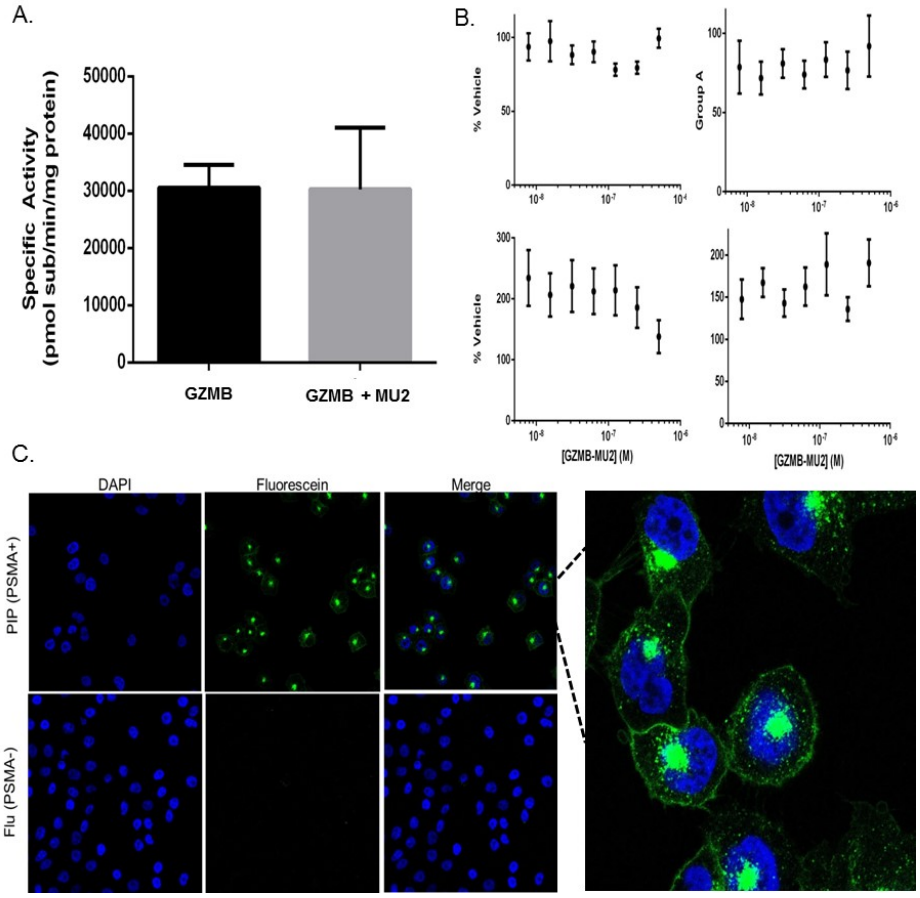


Figure 7: Protein-urea conjugates bind and inhibit PSMA. Scheme of the enzyme-coupled PSMA enzymatic assay utilized to detect urea-conjugate binding (A). Inhibition of PSMA by coupled or naked cytotoxic proteins represented as a percentage of the control reaction (C). Table describing the IC50 values for PSMA obtained for each compound (D).



Compound	Cargo	IC50 For PSMA (nM)
ZJ43	Control Urea	1.94
BSA-MU2	Bovine Serum Albumin	174
GZMB-MU2	Human Granzyme B	58.7

Figure 8: GZMB-MU2 internalizes into PIP cells but does not induce cell death. Enzymatic activity of GZMB or GZMB-MU2 using a GZMB-specific fluorescent substrate (A). Cytotoxicity of purified GZMB-MU2 on PIP-PC3 (top left), Flu-PC3 (top right), LAPC4 (bottom left), or CWR22 Rv1 (bottom right) (B). Confocal microscopy of PIP or Flu-PC3 cells treated with Flor-GZMB-MU2 for 1 hour at 20X magnification (left) or 60X (right) (C).



What opportunities for training and professional development has the project provided?

Training activities: The grant supported the graduate training of Oliver Rogers who is a graduate student in the Pharmacology and Molecular Science Department within the Johns Hopkins University School of Medicine. The project described in this grant represents this student's thesis project. In 2016 the student has presented his data at a 3rd Annual Protein and Antibody Therapeutics meeting in Boston MA and the Prostate Cancer Spore Meeting in Fort Lauderdale as well as at local science meetings at Johns Hopkins including our Fellow Research Day.

How were the results disseminated to communities of interest?

Poster and podium presentations at the meetings describe above.

What do you plan to do during the next reporting period to accomplish the goals?

We will continue to pursue our original objectives. We plan to further characterize the PSA and PSMA activated Granzyme B protoxins in vivo against PSA and PSMA producing prostate cancer xenografts. We will also begin to develop methods for co-targeting by generating a PSA-activated PSMA binding version of Granzyme B. We are also considering other human protein toxins that may be more amenable to a PSMA targeting approach based on their ability to escape from the endosome better than the PSMA-Granzyme B construct.

4. **IMPACT:**

What was the impact on the development of the principal discipline(s) of the project?

Nothing to report

What was the impact on other disciplines?

Nothing to report

What was the impact on technology transfer?

We are working with the Technology Transfer office at Johns Hopkins to submit a Patent application on these findings.

What was the impact on society beyond science and technology?

Nothing to report

5. CHANGES/PROBLEMS:

Changes in approach and reasons for change

Nothing to report

Actual or anticipated problems or delays and actions or plans to resolve them

None

Changes that had a significant impact on expenditures

None

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

Significant changes in use or care of human subjects

Nothing to report

Significant changes in use or care of vertebrate animals

Nothing to report

Significant changes in use of biohazards and/or select agents

Nothing to report

6. PRODUCTS:

- **Publications, conference papers, and presentations**
  Report only the major publication(s) resulting from the work under this award.

  Journal publications.

A manuscript describing the PSA-Granzyme B studies is in preparation. Otherwise nothing to report.

Books or other non-periodical, one-time publications.

Nothing to report

Other publications, conference papers and presentations.

See above for description of presentations

- **Website(s) or other Internet site(s)**

Nothing to report

- **Technologies or techniques**

Nothing to report

- **Inventions, patent applications, and/or licenses**

Nothing to report

- **Other Products**

Nothing to report

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Name:	*Samuel Denmeade*
Project Role:	*PI*
Researcher Identifier (e.g. ORCID ID):	*NA*
Nearest person month worked:	*3*
Contribution to Project:	*Dr. Denmeade is the PI who has designed and oversighted all of the work described in the proposal*
Name:	*Oliver Rogers*
Project Role:	*Graduate Student*
Researcher Identifier (e.g. ORCID ID):	*NA*
Nearest person month worked:	*12*
Contribution to Project:	*Mr. Rogers has performed all of the laboratory studies described in this proposal under the supervision of Dr. Denmeade.*

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

Nothing to Report

What other organizations were involved as partners?

Nothing to Report

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS: F**N/A**

QUAD CHARTS: N/A

9. APPENDICES: N/A